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DIFFERENTIATION OF AMINO ACIDS BY GAS-LIQUID CHROMATOGRAPHY OF THEIR PYROLYSIS PRODUCTS

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DIFFERENTIATION OF AMINO ACIDS BY GAS-LIQUID CHROMATOGRAPHY OF THEIR PYROLYSIS PRODUCTS

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ABSTRACT

Conditions are described for the low-temperature pyrolysis of amino acids and gas-liquid chromatography of the amines produced. Pyrolysis is accomplished at 300°C, and the amines are stabilized at 110°C prior to chromatography on Quadrol. Aliphatic amines C_1 to C_5 can be observed under these conditions. Each amino acid gives a unique amine profile, and proteins give reproducible amine profiles related to their amino acid content.

CONTENTS

FIGURES

TABLES

I. INTRODUCTION

Amino acids of protein hydrolyzates, usually identified by paper chromatography or electrophoresis,[1] may be quantitatively analyzed by ion exchange techniques,[2,3] gas chromatography of their volatile derivatives,[4-6] or by gas chromatography of their catalytic oxidation products.[7] This report describes a technique for the study of amino acids by gas chromatography of their low-temperature pyrolysis products. Evidence indicates that this technique may be applied to the identification of specific proteins and other nitrogenous material.

II. EXPERIMENTAL WORK

A. APPARATUS

The glass fiber filter paper used in these experiments was obtained from Mine Safety Appliances Company, Pittsburgh, Pennsylvania. The amino acids and peptides were purchased from the California Corporation for Biochemical Research, Los Angeles 34, California, the Mann Research Laboratories, Inc., New York 6, New York, and the Nutritional Biochemicals Corporation, Cleveland, Ohio. The proteins were obtained from Nutritional Biochemicals Corporation, Merck and Company, Rahway, New Jersey, and from Difco Laboratories, Detroit, Michigan. The gas chromatograph was an F & M Model 500 with a tungsten filament detector. The pyrolysis chamber was designed by the authors for simple use with the Model GV-11 gas sampling valve of this instrument, and is diagrammed in Figure 1.

B. COLUMN PREPARATION

Unpublished work at the U.S. Army Biological Laboratories* indicated that the main nitrogenous pyrolytic products of protein at a temperature of approximately 300°C are ammonia and aliphatic amines. Many problems are encountered in the gas chromatography of amines,[8] including "tailing" due to adsorption on the solid support and to the extensive solubility of amines in the water produced during pyrolysis. Water tends to condense in the connective tubing of the gas sampling valve, and may prevent some of the amine material from ever reaching the column. Moreover, water usually is eluted from the column in the region of interest, thereby obscuring some of the amine peaks on the chromatogram.

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* Randall, G. U.S. Army Biological Laboratories, personal communication.

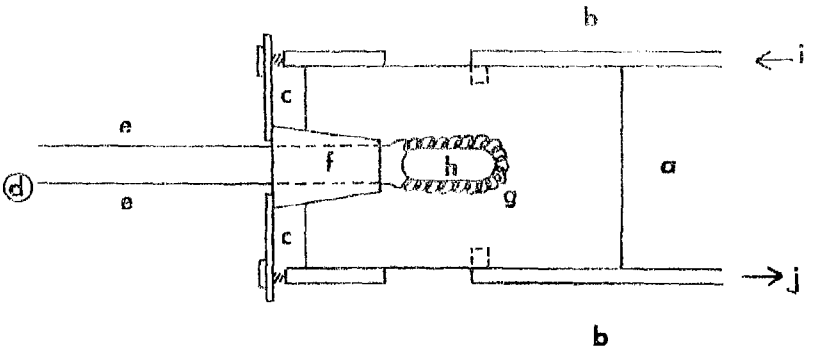

Figure 1. Pyrolysis Chamber.
a = 1" x 1" x 1½" stainless steel chamber
b = 1/8" O.D. stainless steel tubing
c = clamp for plug
d = ammeter
e = 18-gauge copper wire leads
f = Teflon plug
g = tungsten filament
h = sample
i = carrier gas in
j = carrier gas out

Condensation in the connective tubing was minimized by treating the inside surface with hexamethyldisilazane, and by maintaining the temperature of the tubing above 100°C with a heating tape. Several methods for deactivating the solid support and retarding the elution of water have been previously suggested.[8,9] These were found unsatisfactory for the present purposes.

Columns of 0.75 per cent silicone gum rubber and of 20 per cent triethanolamine on Anakrom ABS were found completely unsatisfactory; amines tailed badly and water interfered. Thirty per cent of a mixture of 15 per cent Nujol and 85 per cent hendecanol on Gas Chrom P[9-11] was satisfactory for ammonia and methyl amines, but the useful life of the column (about two weeks) was too short for routine use.

It was found that columns of five per cent DC silicone oil 710 or five per cent silicone oil 200 on methanolic KOH-washed Chromosorb W, programmed from 100°C to 200°C at 7.9 degrees per minute, were excellent for the separation of C_4 to C_9 aliphatic amines and pyridine homologues. However, few amines above C_4 were produced in the amino acid pyrolysates.

1. Column Packing

The column packing found most satisfactory for separation of air, ammonia, and C_1 to C_5 aliphatic amines with no interference from water was prepared as follows:

Five grams of reagent-grade KOH were dissolved in enough distilled water to slurry 95 grams of silanized Chromosorb P (120/140 mesh). After drying at 110°C, the KOH-coated Chromosorb was slurried with 17.7 grams of Quadrol [tetrakis (2-hydroxypropyl) ethylenediamine] in a five per cent solution of chloroform in light petroleum ether. This provided 0.15 gram of Quadrol per gram of packing. After the solvent evaporated, the packing was cured at 105°C overnight and packed into a copper or stainless steel tube six feet long and four millimeters inside diameter. The ends of the tube were secured with small plugs of hexamethyldisilazane-treated Pyrex glass wool to avoid amine adsorption at this often-neglected point. If copper tubing is used, its inside surface should also be silanized. No decomposition of amines was observed when this was done. This column was operated at 70°C, but the temperature was raised to 100°C between analyses to remove adsorbed water.

2. Conditions for Pyrolysis

Pyrolyzer - see Figure 1
Volts - 6 a.c.
Amperes - 6
Pyrolysis Time - 3 minutes
Filament Temperature - 950°C based on color
Chamber Atmosphere - helium
Injection (chamber flush) - 10 seconds onto column

3. Conditions for Gas Chromatography

Column - 15% Quadrol over 5% KOH on silanized Chromosorb P, 120/140 mesh
Length - 6 ft.
Inside Diameter - 4 mm.
Carrier Gas - Helium filtered through Linde Molecular Sieve 13X
Column Temperature - 70°C
Detector Temperature - 125°C
Detector Current - 150 mamp.

C. PROCEDURE

A 30-milligram sample of amino acid or protein was wrapped in glass fiber filter paper and inserted into the filament of the pyrolyzer as shown in Figure 1, rather than being coated on the filament in the usual manner.[12,13] The wrapping insured that the sample would not be driven off the filament before pyrolysis was complete. The chamber was tested for leaks and purged with carrier gas while the column was held at 105°C to prevent the adsorption of moisture from the air in the chamber. The resulting helium atmosphere in the chamber prevented the oxidation of the amines to nitrogen oxides, which would interfere with the analysis.

After the chamber was purged and the carrier gas diverted past it, the column temperature was readjusted to 70°C. This temperature gave the best resolution of the amines studied. The sample was then heated to 300°C as determined by a thermocouple probe inserted into the filter paper, and current was maintained through the filament for three minutes to complete the evolution of degradation products and to allow for equilibration of the gases at the temperature of the chamber atmosphere (110°C). This equilibration allowed interaction of the vapors, producing a more complex amine mixture than would have been stable at the filament temperature. It was also found necessary for reproducible profiles. Slight variations in filament temperature were found to significantly affect the quantitative yield of amines, but the qualitative yield was constant over a range of about 100 degrees (Figure 2).

The carrier gas stream was channeled through the pyrolysis chamber, flushing its contents onto the column for ten seconds. The gas stream was then re-routed past the chamber. Although this did not completely empty the chamber of pyrolysate vapors, it gave the maximum sample size that would not overload the column.

The amines were identified by comparison of their relative retention volumns (tripropylamine = 1.00) with those of known standards analyzed under the same chromatographic conditions (Table I). The relative retention volumns obtained from the pyrolysate amines were corrected for the delay in moving from the pyrolysis chamber to the column. The effluent from the column side of the detector was passed into methyl red or Nessler's reagent to confirm the presence of amines.

To determine if this technique could be applied to proteins and protein mixtures, samples of crystalline egg albumin, bovine serum albumin, glutathione, histidylhistidine and lyophilized cells of Sarcina lutea were pyrolyzed under conditions identical to those used on individual amino acids. Results are shown in Table II.

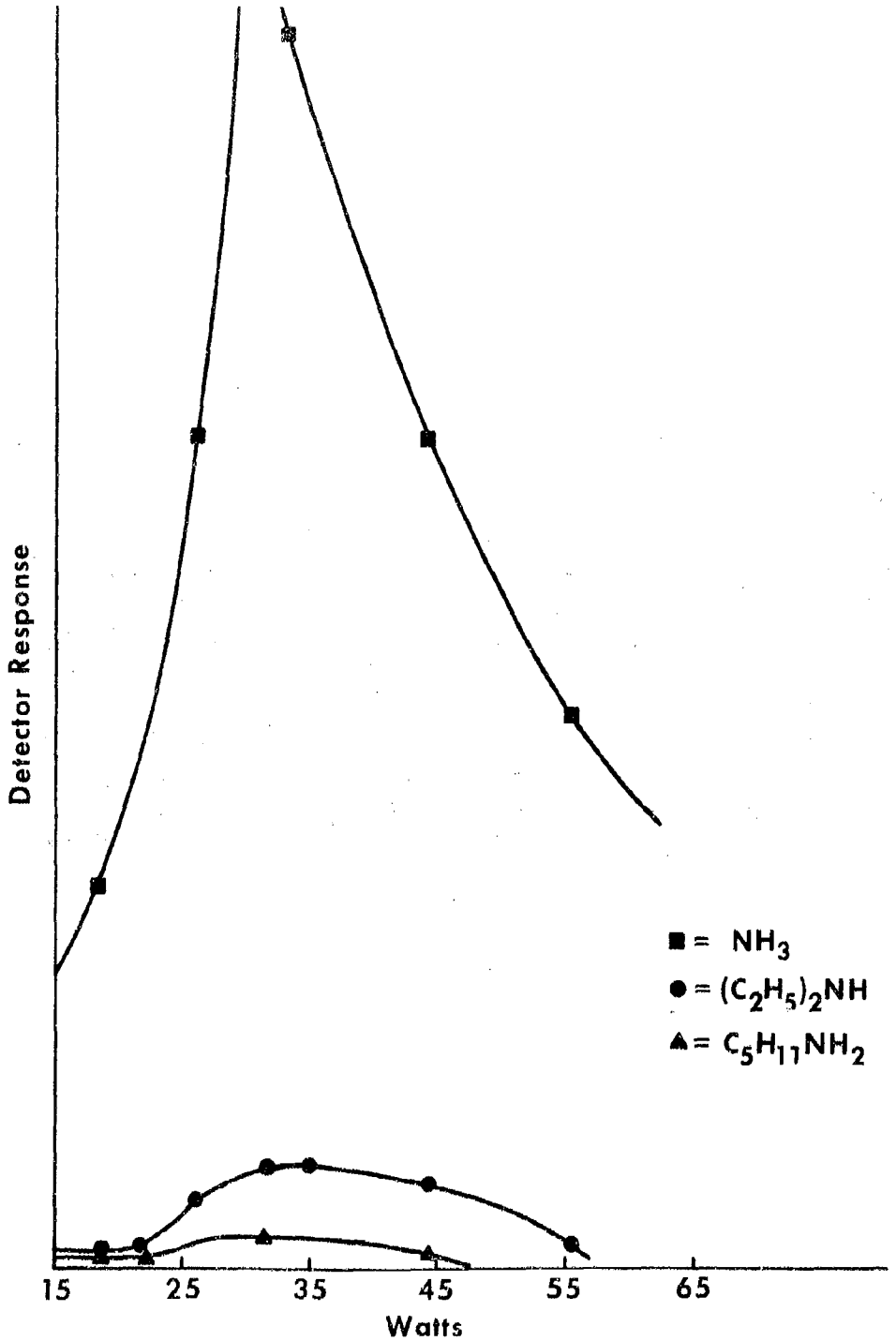

Figure 2. Effect of Filament Power on Evolution of Amines from Egg Albumin.

TABLE I. IDENTIFICATION OF PEAKS IN TABLES

Peak	Compound	Relative Retention Volume[a]
A	Ammonia	0.42
B	Methyl and Dimethyl Amines	0.60
C	Ethylamine	0.82
D	unidentified	0.92
E	Tripropylamine and/or Benzene	1.00
F	Dipropylamine	1.25
G	Tributylamine	1.46
H	Butylamine	1.96
I	Dibutylamine	3.66
J	unidentified	3.16
K	unidentified	2.08
L	Amylamine	2.37

a. Tripropylamine set equal to 1.00.

TABLE II. AMINE PROFILES FOR PROTEINS AND PEPTIDES

	Average Peak Height, inches					
Peak	Egg Albumin	Bovine Albumin	Hemoglobin	Histidyl-histidine	Glycyl-glycine	Glutathion
A	16.3	40.0	27.2	40.0	45.6	12.8
B	2.0	1.8	1.0	-	-	1.2
C	0.5	4.2	-	-	-	-
D	4.9	-	3.5	-	-	2.5
E	4.2	-	1.7	1.8	7.2	6.1
F	-	4.4	-	-	-	-
G	0.2	0.1	-	0.3	-	-
H	0.1	-	0.2	0.1	-	-
I	-	-	-	-	-	-
J	-	-	-	-	-	-
K	-	-	-	-	-	-
L	-	-	-	-	-	-

An attempt was made to relate the amine profile of S. lutea to the amino acids actually present. One gram of lyophilized S. lutea cells was hydrolyzed in 6N HCl under reflux for three hours. The hydrolyzate was analyzed by paper chromatography against known standards. N-butanol/glacial acetic acid/water, 2 : 1 : 1, and isopropanol/con. HCl/water, 65.0 : 16.6 : 18.4 were used as solvent systems according to the method of Fink, Klein and Fink.[14] Serine, cystine, leucine, glutamic acid, phenylalanine, alanine, and tyrosine were found present. Cystine, serine, and leucine appeared to be the major amino acids released in this brief hydrolysis.

Samples of crystalline egg albumin and bovine serum albumin were pyrolyzed as described above (Figure 3). An attempt was made to relate the amine profiles obtained from these proteins to the amino acids they contain. The amount of each amine obtained from each amino acid (when pyrolyzed separately), was multiplied by the per cent of that amino acid in the particular protein being studied.[15] If the amount of each amine in the protein pyrolysate was directly related to the sum of the contributions to that amine made by each amino acid releasing it, summing the (amount) x (%) values obtained above should give a value equal or proportional to the amount of that amine in the protein pyrolyzate. The results are summarized in Table III.

To further test the possibility that each amino acid when pyrolyzed in a mixture yields the same amines in the same proportions that it yields when pyrolyzed separately, mixtures of phenylalanine-valine 1:1 (w/w) and phenylalanine - methionine 1:1 (w/w) were pyrolyzed (Figure 4).

III. RESULTS AND DISCUSSION

Under the conditions described, each amino acid or protein gave a unique and reproducible amine profile, as set forth in Tables II and IV. No amines were observed having carbon chains longer than those present in the parent amino acid. However, the presence of di- and tri- amines indicates that mere degradation of the amino acid does not account for all of the amines observed. Interaction and recombination probably take place in the cooler portions of the chamber.

Several peaks appeared that did not correspond in relative retention volume to any symmetrical amine standard. Some of the unidentified peaks, for example peak J (Table IV) from leucine, elute from the column at points expected for iso-amines. Since one amine usually present in each elution profile is equivalent to the parent amino acid less the carboxly group,

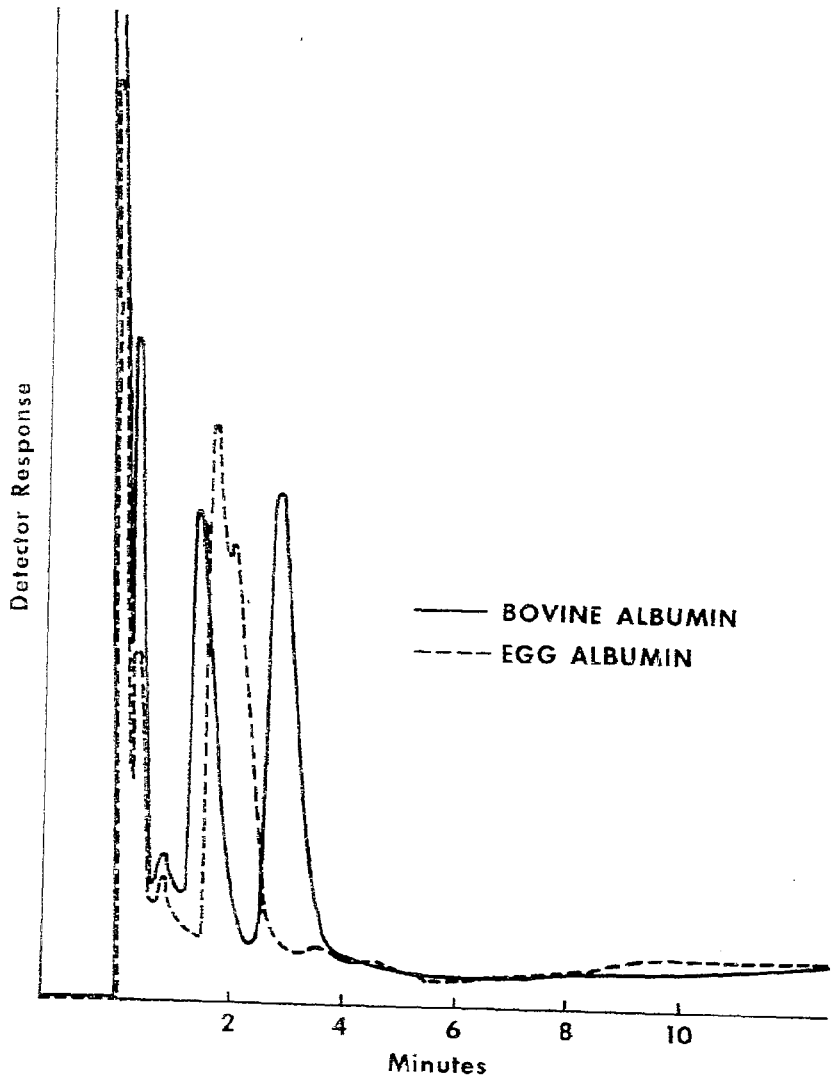

Figure 3. Chromatograms of Egg and Bovine Serum Albumin Pyrolysates.

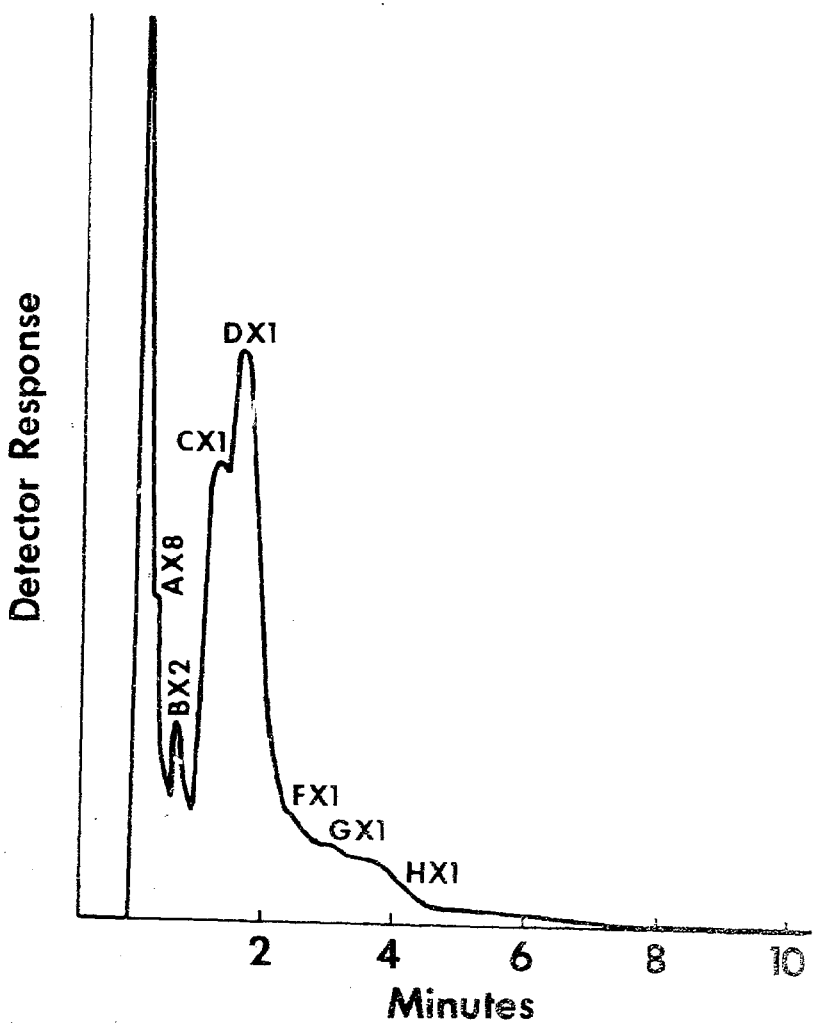

Figure 4. Chromatograms of Phenylalanine-Methionine Mixture Pyrolysate.

TABLE III. COMPARISON OF COMPUTED AND MEASURED PEAK HEIGHTS FOR EGG ALBUMIN PYROLYSATE

Peak	Sample 1	Sample 2	Sample 3	Average	Computed
A	18.4	16.0	14.6	16.3	16.2
B	2.2	1.8	NR[a/]	2.0	2.0
C	0.7	0.5	0.4	0.5	0.4
D	5.1	4.8	4.9	4.9	4.0
E	4.0	4.4	4.3	4.2	4.2
F		NOT RESOLVED FROM E		-	0.8
G	0.2	0.3	0.0	0.2	0.1
H	0.1	0.1	0.1	0.1	0.1
I	0	0	0	0.0	0.0
J	0	0	0	0.0	0.0
K	0	0	0	0.0	0.0
L	0	0	0	0.0	0.0

a. NR = not resolved to automatic attenuation on recorder.

TABLE IV. AMINE PROFILES OF AMINO ACID PYROLYSATES

Amino Acid	Peak Height, inches[a]											
	A	B	C	D	E	F	G	H	I	J	K	L
Phenylalanine	18.8	1.9	7.0	-[b]	5.3	1.6	0.2	-	-	0.5	-	-
Glycine	53.8	-	-	-	6.1	-	-	-	-	-	-	-
Serine	30.0	-	-	1.1	5.6	0.7	0.2	-	-	-	-	-
Asparagine	76.0	1.3	-	-	1.6	-	-	-	-	-	-	-
Norleucine	22.4	1.3	1.0	0.4	0.9	0.4	-	-	-	0.6	-	-
Cystine	12.0	-	-	1.4	-	4.5	-	-	-	-	-	-
Threonine	24.0	5.2	1.2	1.1	0.9	0.2[c]	0.3	-	-	-	-	-
Aspartic Acid	1.3	-	-	-	-	0.8	-	-	-	-	-	-
Glutamic Acid	-	1.1	-	3.0	3.1	-	-	-	-	-	-	-
Alanine	24.0	-	-	27.2	14.0	-	0.4	-	-	-	-	-
Valine	16.0	18.0	-	2.6	6.8	-	-	-	-	-	0.5	-
Isoleucine	8.0	0.1	-	0.4	3.7	0.5	0.6	-	-	-	0.7	-
Leucine	16.0	1.2	0.6	1.2	4.3	1.0	-	-	-	1.3	-	-
Proline	3.0	1.0	-	-	0.5	-	-	-	-	-	-	-
Tryptophan	-	1.9	-	2.1	-	-	-	-	-	-	-	-
Lysine	1.6	1.6	1.5	6.1	1.2	0.2	0.3	-	-	-	-	-
Methionine	38.4	1.5[c]	2.5	6.1	11.6	-	-	-	-	-	-	-
Tyrosine	30.8	1.6	-	0.9	-	0.1	-	-	-	0.1	0.1	-

a. From 30-milligram amino acid samples, average of three determinations.
b. Peak absent.
c. Occasionally much higher; very temperature-sensitive.

the structure of leucine suggests that isobutyl amine should be present in its pyrolysate. However, positive identification of this peak can not be made in the absence of known iso-amine standards, especially since peak J also results from the pyrolysis of tyrosine, which contains no iso groups.

The amines found in the pyrolysate of S. lutea included all the amines observed in the profiles of the amino acids known to be present. No other peaks appeared.

It is apparent from an examination of Table III that the amine profile obtained from egg albumin is related to the individual amine profiles of the component amino acids. In the case of some of the amines, the relationship appears to involve simple addition of the amounts of those amines that would be produced from each amino acid if pyrolyzed separately. In other cases, for example peak D, the relationship does not appear to involve simple addition. One fact involved in this observation may be that benzene, a possible pyrolytic product of phenylalanine and tyrosine, is also eluted at peak D.

Several samples of egg albumin, each from a different source, all gave qualitatively identical amine profiles. However, Figure 3 shows that even proteins of the same general class, in this case egg and serum albumins, exhibit significant qualitative differences when analyzed by this technique. It is interesting to note that peak F is seen to be much more conspicuous in the serum albumin profile than in that of egg albumin. When pyrolyzed alone, cystine yields large quantities of amine eluting at peak F, and cystine makes up four times as much of the amino acid content of serum albumin as it does of egg albumin.[16]

When a mixture of phenylalanine and methionine was pyrolyzed, the resultant amine profile was qualitatively similar to a combination of the profiles obtained by pyrolyzing phenylalanine and methionine separately. However, the amounts of each amine in the pyrolyzed mixture were not equal to the sums of the amounts that would have been obtained by separately pyrolyzing the components (Table V). Moreover, when the phenylalanine-valine mixture was pyrolyzed, the resulting amine profile contained amines not found in the pyrolysates of either individual amino acid.

A comparison of the results of pyrolyzing glycine (Table IV) with those of pyrolyzing glycylglycine (Table II) shows that the two amine profiles are qualitatively similar, and moreover that the ratio of total amine from glycylglycine to total amine from glycine is directly proportional to the ratio of their nitrogen contents.

TABLE V. EFFECT OF PYROLYZING A MIXTURE

Peak	P*	M+	½(P+M)	Peak Height, Mixture x
A	18.8	38.4	27.6	13.1
B	1.9	27.2	14.5	11.2
C	7.0	2.5	4.8	4.5
D	-	5.2	2.6	2.2
E	5.3	10.6	8.0	9.0
F	10.1	-	5.1	5.3
G	0.2	-	0.1	0.15
H	-	-	-	-
I	-	-	-	-
J	0.5	-	0.25	0.2
K	-	-	-	-
L	-	-	-	-

* Peak heights for 30 milligrams phenylalanine.
\+ Peak heights for 30 milligrams methionine.
x Actual peak heights for mixture of 15 milligrams phenylalanine and 15 milligrams methionine.

Every substance containing amino acid so far tested by this method has given a unique amine profile. Although some information can be deduced from this profile as to the structure and composition of the substance pyrolyzed, this technique is presently most useful as a means of identifying particular nitrogenous compounds. With further theoretical study and refinements for quantitative work, this technique may be found useful in the future study of such incompletely characterized substances as enzymes, nucleic acids, and antibodies.

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